

INTERINDIVIDUAL VARIATION IN MITOCHONDRIAL ENZYME ACTIVITY IN MALE MONARCH BUTTERFLIES, *DANAUS PLEXIPPUS* L. (NYMPHALIDAE)

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ABSTRACT. We determined activity levels for the mitochondrial enzyme succinate dehydrogenase in male monarch butterflies overwintering on the central coast of California from October 2001 through March 2002. Mitochondrial activity is important for generating the energy required for metabolically demanding activities such as flight, which is essential for monarch reproduction and survival. There is a genetic component to variation in flight performance in various species, and individual variation in mitochondrial activity may contribute to these differences, since mitochondrial enzyme levels often correlate with performance abilities. To understand possible functional consequences of mitochondrial activity, it is first necessary to determine the degree of individual variation within the population. We found a high degree of interindividual variation in enzyme activity in male monarchs, at least a twelve-fold difference between the lowest and highest activities measured, with a coefficient of variation of forty-seven percent. In addition, we investigated possible correlations with season, body weight, body size, and wing damage. Although there were some month-to-month differences, individual variation in mitochondrial enzyme activity is not explained by seasonality or body size, and is not related to the degree of wing damage. The results suggest that interindividual differences in mitochondrial enzyme activities are considerable, and worth investigating as a factor in individual performance and success.

Additional key words: succinate dehydrogenase, insect flight muscle, overwintering, energetics.

Monarch butterflies are known for their dramatic migration across the U.S.A. and overwintering in massive aggregations along the California coast and in central Mexico (Tuskes & Brower 1978, Brower 1985). Flight is energetically expensive; indeed, insect flight muscle is notable for the highest metabolic rates and power output of any animal tissue (Sacktor 1976, Suarez 2000). In addition to long flights of migration during fall and spring which may cover thousands of miles (Brower 1985), mating involves a short energetically intense nuptial flight. Nuptial flight or mate transport to the nearby tree canopy occurs after a male successfully couples with a female (Shields & Emmel 1973). Regulation of metabolism is also important for survival, since monarchs rely primarily on stored lipids during the overwintering period (Chaplin & Wells 1982, Masters et al. 1988, Alonso-Mejia et al. 1997). Understanding metabolic variation in monarchs could therefore provide valuable insight into differences in flight performance, reproductive success and survival.

Relatively little is known about monarch flight muscle metabolism or about intraspecific metabolic variation in general. Individual variation in flight performance has been shown to be partly genetic in moths (Parker & Gatehouse 1985). Correlations between flight activity or capacity and metabolic differences have been seen in *Agrotis ipsilon* moths (Sappington et al. 1995), *Colias* spp. butterflies (e.g., Watt et al. 1983), *Epiphyas postvittana* moths (Gu 1991) and *Drosophila melanogaster* flies (Barnes & Laurie-Ahlberg 1986, Marden 2000). In *Colias* butterflies, genetic differences in metabolic enzymes also correlated with differing mating success by males (Watt et al.

1985). There is evidence that monarch butterflies may have individual differences in flight ability at cool temperatures (Hughes et al. 1992). Further investigation of metabolic parameters may reveal some of the mechanisms underlying this individual variation.

Mitochondrial activity is a critical aspect of metabolism; insect flight muscle metabolism is strongly aerobic, so most of the energy is produced by the mitochondria (Sacktor 1976). Mitochondrial enzyme levels appear to be a good indicator of mitochondrial density and overall aerobic metabolism measured by oxygen consumption or oxidative capacity in a variety of organisms (e.g., Holloszy & Booth 1976, Spina et al. 1996, Putnam & Bennett 1983). The enzymes most commonly used as markers of mitochondrial activity are succinate dehydrogenase (SDH) and citrate synthase (CS), mitochondrial-specific enzymes with critical roles in the tricarboxylic acid cycle and therefore directly involved in the generation of ATP.

Individual mitochondrial enzyme activities can vary considerably and the variation often correlates with performance. The largest interindividual variation reported is an approximately twenty-fold difference seen in CS activity in leg muscles of 20 toads (*Bufo marinus*) (Longphre & Gatten 1994). More commonly, differences of three- to five-fold are seen in CS activity among individuals in a variety of non-insect species (e.g., Walsberg et al. 1986, Garland & Else 1987). Less is known about individual variation in insect mitochondrial activity, but in one study including 19 insect species, citrate synthase in flight muscle varied as much as three-fold even with only three individuals from each species (Alp 1976). Functional correlates of variation in mitochondrial enzyme activities have been demonstrated in a variety of organisms, most com-

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monly in terms of response to exercise. In a variety of mammals, amphibians and reptiles, correlations have been seen between mitochondrial activity and maximum aerobic speed, endurance, and degree of improvement after exercise training (Longphre & Gatten 1994, Garland & Else 1987, Cummings 1979).

Variables which might be related to mitochondrial activity include seasonality and physical characteristics. Seasonal variation in mitochondrial enzyme activities has been reported in several species, including agamid lizards (Garland & Else 1987), iguanid lizards (John-Alder 1984), turtles (Olson 1987) and snapper (Majed et al. 2002); in all cases the changes are thought to reflect seasonal patterns of activity and energetic requirements. In several organisms including lizards and fish, mitochondrial enzyme activity is related to body size (Garland & Else 1987, Somero & Childress 1980). Wing damage may be related to activity levels, as more damage is likely to occur during activities such as mating. Higher wing damage has been seen in male monarchs attempting to mate or drinking dew, as compared to those remaining in clusters (Frey et al. 1998, Oberhauser & Frey 1999, Frey et al. 2002). It is possible that these males represent a more active subset of the population, which could be related to metabolic differences such as mitochondrial enzyme activity.

Characterization of mitochondrial activity in overwintering monarchs in central California will provide basic information about metabolic variation in this population, which may be important for understanding critical variables for successful overwintering and reproduction. The purposes of this study were to determine the extent of variation in succinate dehydrogenase (SDH) activity in the male overwintering monarch population and investigate possible correlations with season or physical characteristics.

MATERIALS AND METHODS

This study was carried out at an overwintering site at Pismo Beach State Park, Pismo Beach, California. Male monarch butterflies were collected monthly at approximately 0800 h on the following dates: 20 October, 26 October, 21 November, and 16 December 2001; 17 January, 19 February and 14 March 2002. In all cases this was before the temperature reached the flight threshold, so all monarchs were still in clusters (Frey et al. 2002). Butterflies were collected from clusters using a standard butterfly net on a long pole, and 20 males were randomly selected from the net. The butterflies were placed in small ziplock bags and stored in a styrofoam cooler with icepacks until they reached the lab at California Polytechnic State University, approximately fifteen miles away (Frey et al.

2002). At the lab we measured weight to the nearest milligram and forewing length to the nearest millimeter from the thorax to the longest extension on the forewing. We recorded the number of damaged wings containing rips, punctures, or parts missing, and assigned each butterfly a wing condition value of 1–4 based on lack of scales, brightness of color, and damage, with 1 being the worst condition and 4 the best. Each butterfly was assigned an identification number and placed in a ziplock bag to be stored at -60°C for an average of 29 days. There was no relationship between storage time and enzyme activity (regression analysis, $R^2 = 4.8\%$, $n = 118$).

Succinate dehydrogenase (SDH) activity was measured in a sample of thorax muscle tissue from each butterfly using the rate of reduction of the artificial electron acceptor 2,6-dichlorophenolindophenol (DCIP) by procedures modified from Singer and Kearney (1957). Muscle was obtained by removing all appendages from the thorax, freezing it in liquid nitrogen, and then removing 9–11 mg thorax muscle. The tissue sample was weighed and then homogenized with a ground-glass homogenizer for 5 minutes on ice in 250 μl homogenizing buffer (0.3 M mannitol, 0.02 M phosphate, pH 7.2). The homogenate was centrifuged at 600 g for 10 minutes at 4°C to remove particulates and large organelles. Each butterfly tissue was assayed in triplicate, using ninety-six well microplates and a SPECTRAMax Microplate Spectrophotometer (Molecular Devices Corp., Sunnyvale CA). Reactions were carried out in a total volume of 200 μl with 30 μl tissue homogenate, 1.5×10^{-4} M DCIP, 0.002 M sodium azide and 0.01 M sodium succinate in assay medium (0.3 M mannitol, 0.02 M phosphate, 0.01 M potassium chloride, 0.005 M magnesium chloride, pH 7.2). Reactions were started by adding diluted homogenate to the other reagents, and a kinetic assay was immediately run at 600 nm every 15 seconds for 10 minutes. Only the initial linear data was used for calculations, typically the first 3 minutes. SDH activity in $\mu\text{moles}\cdot\text{min}^{-1}\cdot\text{g tissue}^{-1}$ was calculated from the slope of the initial reaction, the extinction coefficient of DCIP (19,100), the measured path length, and the volumes and weights used (activity = slope \times $1/e \times 1/b \times 60\text{sec}/\text{min} \times$ assay volume/sample homogenate vol \times total homogenate volume/tissue weight \times 1000 mg/1g). All statistical analyses were performed using Minitab (Minitab Inc.).

RESULTS

Succinate dehydrogenase (SDH) activity was determined for a total of 120 butterflies. The overall distribution of SDH activity for the overwintering season

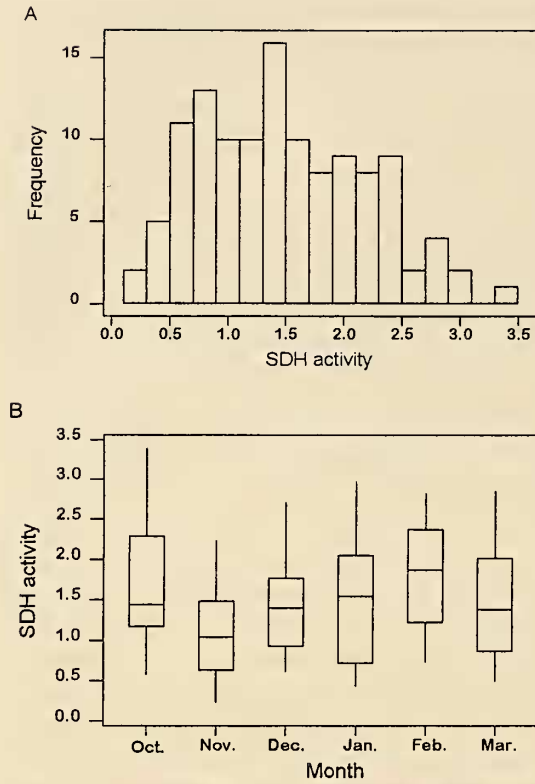


FIG. 1. SDH activity in male overwintering monarchs. SDH activity is given as $\mu\text{moles}\cdot\text{min}^{-1}\cdot\text{g tissue}^{-1}$. **A**, Frequency distribution of SDH activity for the entire sampled population. **B**, Boxplot of monthly SDH activity: the horizontal line represents the median and the ends of the box represent the upper and lower quartiles.

is shown in Fig. 1A, and ranged from 0.23–3.40 $\mu\text{moles}\cdot\text{min}^{-1}\cdot\text{g tissue}^{-1}$, approximately a fifteen-fold difference. Ninety percent of the values fall between 0.5 and 2.7 $\mu\text{moles}\cdot\text{min}^{-1}\cdot\text{g tissue}^{-1}$, a five-fold range. Since SDH activity was measured in triplicate samples from each butterfly, the standard error associated with the mean SDH value for each gives some indication of the analytical error; the mean standard error is 0.16, approximately eleven percent on average. Given this degree of analytical uncertainty, a conservative estimate still yields at a minimum a four-fold range for ninety percent of the enzyme activities and a twelve-fold range for all values. To illustrate the degree of variation in SDH activity, we calculated the coefficient of variation ($CV = SD \cdot \bar{x}^{-1} \cdot 100$). The overall CV for the population over the entire overwintering period was forty-seven percent while monthly CVs ranged from thirty-six percent in February to fifty-two percent in November (Table 1).

Monthly distributions of SDH activity are shown in Fig. 1B and Table 1; there was clearly considerable variation within each month. Season does have a significant association with SDH activity, as seen in a one-

TABLE 1. Basic statistics for SDH activity, body weight, and wing length. SDH activity is given as $\mu\text{moles}\cdot\text{min}^{-1}\cdot\text{g tissue}^{-1}$. Coefficient of variation (CV) is the standard deviation divided by the mean, multiplied by 100. Subscripts (a, b) for SDH activity means are based on Tukey's pairwise comparisons; the only significant differences are between October and November, and November and February. Sample sizes are $n = 20$ for each month except for February $n = 19$.

	Mean	SD	Mini- mum	Maxi- mum	CV (%)
SDH activity					
October	1.73 _a	0.77	0.57	3.40	44
November	1.07 _b	0.55	0.23	2.23	52
December	1.41 _{a,b}	0.58	0.60	2.72	41
January	1.43 _{a,b}	0.73	0.43	2.98	51
February	1.80 _a	0.66	0.73	2.82	36
March	1.42 _{a,b}	0.71	0.49	2.86	50
All butterflies	1.48	0.70	0.23	3.40	47
Body weight (mg)					
October	599	80	454	692	13
November	536	68	490	705	13
December	529	73	395	745	14
January	539	68	434	670	13
February	557	65	430	660	12
March	478	87	330	650	18
All butterflies	539	81	330	745	15
Wing length (mm)					
October	51.1	3.0	45	56	6
November	50.7	2.5	45	55	5
December	51.9	2.3	46	56	4
January	50.9	2.1	47	55	4
February	48.2	2.8	42	53	6
March	50.1	2.3	45	53	5
All butterflies	50.5	2.7	42	56	5

way ANOVA for SDH activity vs. month, $F = 3.33$, $p = 0.008$ (Table 2). Post-hoc pairwise comparisons revealed that the only months with significant differences are October vs. November and November vs. February (Table 1). In terms of seasonal pattern in the means, we regressed SDH activity on centered month data up to a fifth order polynomial, and only the third order polynomial was significant ($p = 0.001$); however this pattern explains very little of the variation ($R^2 = 9.8\%$).

Differences in the population at the beginning and end of the season may affect the results: in October only about 10% of the butterflies have arrived compared to the peak population in December and January, and by March less than 10% of the butterflies are left at the overwintering site (D. Frey pers. com.). Therefore, we also analyzed the data for the core overwintering season when the population is more complete. Consideration of the centered data only from November through February reveals a linear pattern ($p = 0.001$), but this still only explains a fraction of the individual variation ($R^2 = 13.5\%$). The estimated increase in SDH activity per month is $0.22 \pm 0.06 \mu\text{moles}\cdot\text{min}^{-1}\cdot\text{g tissue}^{-1}$.

TABLE 2. ANOVA for a general linear model of SDH vs. month and all physical variables (body weight, wing length, wing damage, and wing condition). Calculations were performed for the full model and for month alone, with all physical variables removed.

Source	df	SS	MS	F	p
Month	5	7.4036	1.4807	3.33	0.008
Physical variables	4	1.8438	0.4610	1.04	0.391
Error	109	48.4266	0.4443	—	—
Total	118	57.6740	—	—	—

We examined two measures of size (body weight and wing length) and two measures of wing damage (number of damaged wings and overall wing condition) for possible correlation with SDH activity; size data is summarized in Table 1. Mean wet body weight was found to decline from October to March, with considerable variation apart from the linear decrease (simple linear regression analysis, $R^2 = 10.4\%$, $t = 3.69$, $p = 0.000$). Mean wing length fluctuated through the overwintering period and declined slightly in February and March (simple linear regression analysis, $R^2 =$

6.0% , $t = 2.74$, $p = 0.007$). The coefficient of variation was fifteen percent for body weight and five percent for wing length, and the CVs within each month are comparable to the overall population. None of the physical variables contributed significantly to SDH activity (comparison of ANOVA general linear model with all variables to model with only month, $F = 1.04$, $p = 0.391$; Table 2).

DISCUSSION

Interindividual variation in succinate dehydrogenase activity. We found a large degree of interindividual variation in SDH activity in the male monarch population overwintering at Pismo Beach, at least a twelve-fold range of enzyme activities. This degree of variation is greater than that seen in other physical characteristics measured; the coefficient of variation for SDH activity is forty-seven percent compared to a CV of fifteen percent for body weight and five percent for wing length.

Some of the calculated variation in SDH activity

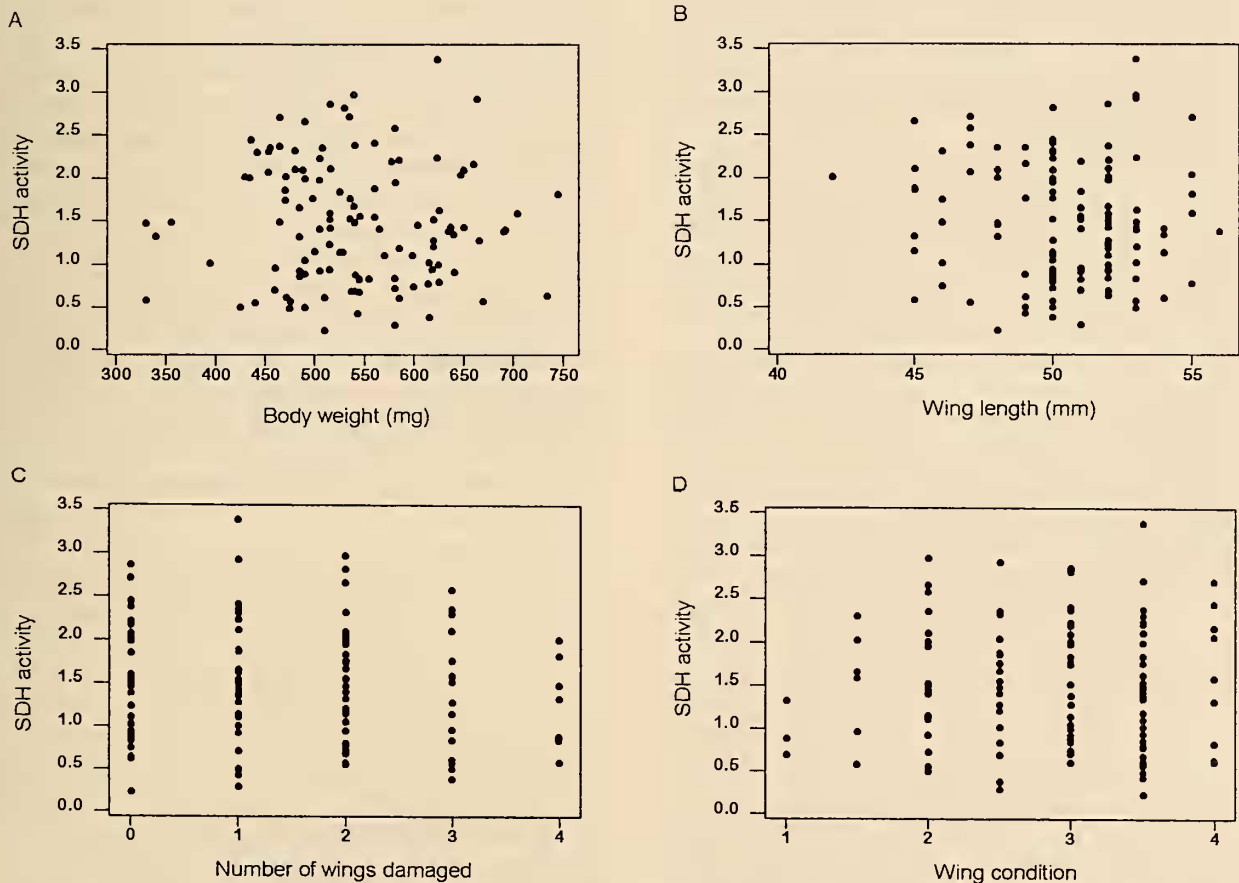


FIG. 2. SDH activity and morphological variables. SDH activity is given as $\mu\text{moles}\cdot\text{min}^{-1}\cdot\text{g tissue}^{-1}$. A, SDH activity and butterfly body weight. B, SDH activity and butterfly wing length. C, SDH activity and the number of wings damaged. D, SDH activity and wing condition on a scale from 1 (worst) to 4 (best).

($\mu\text{moles}\cdot\text{min}^{-1}\cdot\text{g tissue}^{-1}$) may be an artifact of normalizing by tissue weight. Enzyme activities are most commonly normalized by wet tissue weight, but dry tissue weight, protein content, DNA content, and entire organ weight have also been used (Pelletier et al. 1995, Cascarano et al. 1978, Spina et al. 1996, Longphre & Gatten 1994). Monarchs are known to vary in their state of hydration; in Mexico and southern California more dehydrated males are seen at the end of the overwintering season (Chaplin & Wells 1982, Calvert & Lawton 1993), while this pattern has not been seen in central California (D. Frey pers. com.). Individual differences in hydration status may have introduced some variation into individual SDH activity values, but there is no overall correlation between lower body weight and higher SDH activity; such a correlation might be expected if dehydration led to a decrease in body weight but an increase in the number of cells per tissue weight. Normalization by protein content would address this issue but could introduce other problems, since lipid reserves may be depleted at the end of the season leading to a breakdown in protein (Chaplin & Wells 1982, Masters et al. 1988, Alonso-Mejia et al. 1997). Future studies will investigate the effects of different normalization approaches and possible changes in protein content at the end of the overwintering season.

The degree of interindividual variation in SDH activity seen here in male monarchs is within the ranges found in other organisms; more importantly, it is large enough to have functional consequences since as little as a two-fold difference has been correlated with performance differences (Holloszy & Booth 1976).

Relationship between succinate dehydrogenase activity and other variables. Seasonal effects are small in comparison to the large variation in individual SDH activity levels in butterflies collected on the same date. Mean enzyme activity in November is significantly lower than in either October or February, and there are no significant differences between any other months. It is possible that the higher mean SDH activity in October could reflect earlier arrival by a subset of butterflies with higher mitochondrial activity. An increase in mitochondrial activity from November to February could be adaptive since the strenuous exertion of mating occurs late in the season (Tuskes & Brower 1978); an increase could also be a consequence of prior flight activity, analogous to the exercise training effects seen in other studies. The March mean is not significantly different from February, and the downward trend seen could reflect preferential emigration of butterflies with higher mitochondrial activity, since only a small fraction of the overwintering

population remains in March. It is also possible that mitochondrial activities in November were lower than in other months because of unknown environmental factors; preliminary analysis of air temperature data did not reveal any correlations with SDH activity or unusual occurrences in November (data not shown), but careful analysis of potential weather factors has not been done. Further investigation will be necessary to confirm that the seasonal patterns are seen consistently in different years and to determine possible causes.

None of the physical characteristics examined appear to be important in determining mitochondrial enzyme activity in male overwintering monarchs. Other studies have found more linear declines in body weight during the overwintering season, but the degree of weight loss depends on the specific overwintering colony examined (Tuskes & Brower 1978, Chaplin & Wells 1982, Calvert & Lawton 1993), and it is possible that the Pismo Beach site allows better maintenance of body weight. The pattern of wing lengths seen is similar to that reported by Calvert and Lawton (1993) at Mexican overwintering sites except that they observed a more dramatic drop in late February and March after stability for the rest of the season. They suggest that the decrease at the end of the season could be due to larger butterflies leaving the colony first. Our observation that mean body weight is lower in March but wing length is not, suggests that the decreased weight may be due to depleted lipid reserves, dehydration, and/or breakdown of protein in tissues. Since each of these factors could affect mitochondrial enzyme activities, the SDH activity data for March are less easily interpreted than for the rest of the season. The lack of a correlation with wing damage suggests that if there are subsets of the male monarch population with different behaviors and corresponding degrees of wing damage, mitochondrial activity is not a critical determinant nor is it significantly affected by behavioral differences which lead to differing wing damage. Since we have not examined activities directly, it is still possible that differing mitochondrial enzyme levels do affect activity level, performance or mating success.

Mitochondrial activity could be affected by other environmental or physical variables. Age can affect mitochondrial activity and flight performance; changes in mitochondrial structure, an increase in mitochondrial damage and changes in levels of some metabolic enzymes have been reported with aging in the flight muscle of a variety of insect species (Sohal 1976, Ross 2000). We do not have data on the age of the monarchs in this study, but they likely vary by at least a month based on emigration data from late summer popula-

tions (K. Oberhauser pers. com.). However, we found no evidence for an effect of aging on SDH activity since the entire population is aging considerably during the overwintering period and there was not a unidirectional seasonal trend.

Conclusions. The primary conclusion of this study is the high degree of interindividual variation in activity of a mitochondrial enzyme in the male monarch butterflies overwintering at Pismo Beach on the central California coast. This variation is not explained by seasonality or body size and is not related to the degree of wing damage. Individual mitochondrial enzyme activity variation may be partly due to genetic, nutritional, and behavioral (especially in regards to previous activity levels) differences. Mitochondrial activity has potential functional consequences for flight performance, and in the case of monarch butterflies, potential consequences for survival and reproduction. The existence of such substantial individual variation suggests that this could be an important factor in individual performance and success. Future experiments should investigate possible correlations with flight performance and examine mitochondrial activity in female monarchs, especially with regard to energetically demanding reproductive development. In addition, further analysis of metabolic parameters at the end of the overwintering season would provide valuable information about the requirements for successful survival and reproduction during the overwintering period.

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